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(57) Abstract

Pharmaceutical composition and methods of use of an amyloid–beta binding protein from serum and cerebral spinal fluid of Alzheimer's disease and normal aged patients with an estimated size of 350–400 kilodaltons, designated P400. P400 is decreased in serum derived from Alzheimer's disease patients in comparison to normal aged controls. P400 is more abundant than apolipoproteins and laminin in biological fluids and binds Amyloid–beta with a higher affinity.

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Therapeutic and Diagnostic Applications of P400: A Newly Discovered Beta-Amyloid Binding Protein Present in Human Biological Fluids

TECHNICAL FLIELD

The invention relates to the discovery, identification and use of P400 (a newly discovered Aß-binding protein present in human blood and cerebrospinal fluid), P400-derived protein fragments, and P400-derived polypeptides, as well as related peptides and antibodies, for the therapeutic intervention and diagnosis of Alzheimer's disease and other amyloidoses.

BACKGROUND OF THE INVENTION

Alzheimer's disease is characterized by the accumulation of a 39-43 amino acid peptide termed the beta-amyloid protein or Aß, in a fibrillar form, existing as extracellular amyloid plaques and as amyloid within the walls of cerebral blood vessels. Fibrillar Aß amyloid deposition in Alzheimer's disease is believed to be detrimental to the patient and eventually leads to toxicity and neuronal cell death, characteristic hallmarks of Alzheimer's disease. Accumulating evidence implicates amyloid as a major causative factor of Alzheimer's disease pathogenesis.

A variety of other human diseases also demonstrate amyloid deposition and usually involve systemic organs (i.e. organs or tissues lying outside the central nervous system), with the amyloid accumulation leading to organ dysfunction or failure. In Alzheimer's disease and "systemic" amyloid diseases, there is currently no cure or effective treatment, and the patient usually dies within 3 to 10 years from disease onset.

New compounds or agents for therapeutic regimes to arrest or reverse amyloid formation, deposition, accumulation and/or persistence that occurs in Alzheimer's disease and other amyloidoses are therefore desperately sought.

Recent studies indicate that the Aß of Alzheimer's disease is maintained in biological fluids in a soluble state by an unknown mechanism. Implicated proteins which may contribute to such Aß solubility in biological fluids include apolipoproteins, albumin and laminin. In the present invention,

we have encountered a new Aß-binding protein in serum and cerebrospinal fluid (CSF) of Alzheimer's disease and normal aged patients (designated as P400). Our data indicates that P400 in human serum and CSF binds tightly to Aß and is decreased substantially in serum derived from Alzheimer's disease patients in comparison to normal aged controls. These studies suggest the identification of a new Aß-binding protein which may be responsible for the maintenance of Aß solubility in biological fluids. Identification of a new protein which may be a specific indicator for Alzheimer's disease and/or its progression will allow for both novel therapeutic and diagnostic approaches in the future.

SUMMARY OF THE INVENTION

It is known that the Aß of Alzheimer's disease is maintained in biological fluids (i.e. blood and cerebrospinal fluid) in a soluble state by an unknown mechanism. Implicated proteins which may contribute to such maintenance of Aß solubility in biological fluids include apolipoproteins (apolipoproteins J and E)(Ghiso et al, Biochem. J. 293:27-30, 1993; Wisniewski et al, Biochem. Res. Commun. 192:359-365, 1993; Strittmatter et al, Proc. Natl. Acad. Sci. U. S. A. 90:8098-8102, 1993; LaDu et al, J. Biol. Chem. 269:23403-23406, 1994; Naslund et al, Neuron 15:219-228, 1995; Chan et al, Biochemistry 35:7123-7130, 1996), albumin (Biere et al, J. Biol. Chem. 271: 32916-32922, 1996), and laminin (and/or laminin fragments)(Castillo et al, Soc. Neurosci. Abst. 23:1882, 1997). During the course of our studies involving analysis of human biological fluids, we have encountered a new Aß-binding protein in serum and CSF of Alzheimer's disease and normal aged patients, with a size, >205 kilodaltons (and designated P400 due to an approximate estimated size of ~350-450 kilodaltons), which does not appear to be related to any of the previously described proteins above. Our data indicates that P400 binds tightly to Aß (i.e. at subnanomolar concentrations) and is decreased in serum derived from Alzheimer's disease patients in comparison to normal aged controls. In addition, our data suggests that P400 is more abundant than other Aß-binding proteins present in biological fluids including apolipoproteins, and laminin (and/or laminin fragments), and appears to bind Aß with a higher affinity. These studies indicate that we have identified a new Aß-binding protein in biological fluids, which may have diagnostic and therapeutic potential.

FEATURES OF THE INVENTION

A primary object of the present invention is to establish new therapeutic methods and diagnostic applications for the amyloid diseases. The amyloid diseases include, but are not limited to, the amyloid associated with Alzheimer's disease and Down's syndrome (wherein the specific amyloid

is referred to as beta-amyloid protein or Aß), the amyloid associated with chronic inflammation. various forms of malignancy and Familial Mediterranean Fever (wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid is referred to as amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann Straussler syndrome, kuru and animal scrapie (wherein the specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as beta2-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as transthyretin or prealbumin), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred to as variants of procalcitonin).

As disclosed herein, "P400" refers to a newly discovered Aß-binding protein present in human serum and CSF, and which has a molecular weight of approximately 350 to 450 kilodaltons as determined by standard SDS-PAGE (as described herein).

A primary object of the present invention is to use P400, P400 protein fragments and/or P400-derived polypeptides as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses.

Yet another object of the present invention is to use conformational dependent proteins, polypeptides, or fragments thereof for the treatment of Alzheimer's disease and other amyloidoses. Such conformational dependent proteins include, but are not limited to, P400, P400-derived fragments and/or portions thereof.

Yet another aspect of the present invention is to use peptidomimetic compounds modeled from P400, P400-derived protein fragments and/or P400-derived polypeptides, and fragments thereof, as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses.

Yet another object of the present invention is to mimic the 3-dimensional Aß-binding site(s) on P400, P400-derived protein fragments and/or P400-derived polypeptides and use these mimics as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses.

Yet a further aspect of the present invention is to use anti-idiotypic antibodies to P400, P400-derived protein fragments and/or P400-derived polypeptides as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses.

Another aspect of the invention is to provide new and novel polyclonal and/or monoclonal peptide antibodies which can be utilized in a number of in vitro assays to specifically detect Aß-binding P400-derived protein fragments and/or Aß-binding P400 derived polypeptides in human tissues and/or biological fluids. Polyclonal or monoclonal antibodies that are made specifically against a peptide portion or fragment of P400 which interacts with Aß can be utilized to detect and quantify amyloid disease specific P400 fragments in human tissues and/or biological fluids. These antibodies can be made by administering the peptides in antigenic form to a suitable host. Polyclonal or monoclonal antibodies may be prepared by standard techniques known to those skilled in the art.

Another object of the present invention is to use P400, the Aß-binding P400 fragments and/or P400-derived polypeptides referred to above, for the detection and specific localization of P400 peptides important in the amyloid diseases in human tissues, cells, and/or cell culture using standard immunohistochemical techniques.

Yet another aspect of the present invention is to use antibodies recognizing P400, any of the Aß-binding P400 fragments, and/or P400-derived polypeptides, and fragments thereof, for in vivo labeling; for example, with a radionucleotide, for radioimaging to be utilized for in vivo diagnosis, and/or for in vitro diagnosis.

Yet another aspect of the present invention is to make use of P400, P400-derived protein fragments, and P400-derived polypeptides, and fragments thereof, as potential therapeutics to inhibit the deposition, formation, and accumulation of fibrillar amyloid in Alzheimer's disease and other amyloidoses (described above), and to enhance the clearance and/or removal of preformed amyloid deposits in brain (for Alzheimer's disease and Down's syndrome amyloidosis) and in systemic organs (for systemic amyloidoses) .

Another object of the present invention is to use Aß-binding P400-derived polypeptides or fragments thereof, in conjunction with polyclonal and/or monoclonal antibodies generated against these peptide fragments, using in vitro assays to detect amyloid disease specific autoantibodies in human biological fluids. Specific assay systems can be utilized to not only detect the presence of autoantibodies against Aß-binding P400-derived protein fragments or polypeptides thereof in biological fluids, but also to monitor the progression of disease by following elevation or diminution of P400 protein fragments and/or P400-derived polypeptide autoantibody levels.

Another aspect of the invention is to utilize P400, P400-derived protein fragments, and P400-derived antibodies and/or molecular biology probes for the detection of these P400 derivatives in human tissues in the amyloid diseases.

Yet another object of the present invention is to use the P400-derived protein fragments of the present invention in each of the various therapeutic and diagnostic applications described above. Specific P400-derived protein fragments or peptides as described above may be derived from any species including, but are not limited to, human, murine, bovine, porcine, and/or equine species.

Another object of the invention is to provide polyclonal and/or monoclonal peptide antibodies which can be utilized in a number of in vitro assays to specifically detect P400 protein fragments in human tissues and/or biological fluids. Polyclonal or monoclonal antibodies made specifically against a peptide portion or fragment of any of the P400 fragments described herein can be utilized to detect and quantify P400-derived protein fragments in human tissues and/or biological fluids. A preferred embodiment is a polyclonal antibody made to P400 present in human serum and cerebrospinal fluid. These antibodies can be made by isolating and administering P400, P400 fragments and/or P400 polypeptides in antigenic form to a suitable host. Polyclonal or monoclonal antibodies may be prepared by standard techniques by one skilled in the art.

Yet another object of the present invention is to use P400-derived antibodies as described herein as a specific indicator for the presence and extent of P400 breakdown in brain by monitoring biological fluids including, but not limited to, cerebrospinal fluid, blood, serum, urine, saliva, sputum, and stool.

Yet another object of the present invention is to use P400-derived antibodies as described herein as a specific indicator for the presence, extent and/or progression of Alzheimer's disease and/or other brain amyloidoses by monitoring biological fluids including, but not limited to, cerebrospinal fluid, blood, serum, urine, saliva, sputum, and stool.

Yet another object of the present invention is to use P400-derived antibodies as described herein as a specific indicator for the presence and extent of P400 breakdown in systemic organs by monitoring biological fluids including, but not limited to, cerebrospinal fluid, blood, serum, urine, saliva, sputum, and stool.

Yet another object of the present invention is to use P400-derived antibodies as described herein as a specific indicator for the presence and extent of amyloidosis in type II diabetes by monitoring biological fluids including, but not limited to, cerebrospinal fluid, blood, serum, urine, saliva, sputum, and stool.

Yet another object of the present invention is to use P400-derived antibodies as described herein as a specific indicator for the presence and extent of amyloidosis in other systemic amyloidoses by monitoring biological fluids including, but not limited to, cerebrospinal fluid, blood, serum, urine, saliva, sputum, and stool.

Yet another object of the invention is to utilize specific P400-derived antibodies, as described herein, for the detection of P400 in human tissues in the amyloid diseases.

Another object of the present invention is to use P400, P400-derived protein fragments, and P400-derived polypeptides, as described herein, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses.

Another object of the present invention is to use pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, and sterile packaged powders, which contain P400, P400-derived protein fragments, and P400-derived polypeptides, and fragments thereof, to treat patients with Alzheimer's disease and other amyloidoses.

Yet another object of the present invention is to use P400, P400-derived protein fragments, and P400-derived polypeptides, and fragments thereof, as potent agents which inhibit amyloid formation, amyloid deposition, amyloid accumulation, amyloid persistence, and/or cause a dissolution of pre-formed or pre deposited amyloid fibrils in Alzheimer's disease, and other amyloidoses.

Yet another object of the present invention is to provide the use of P400, P400-derived protein fragments, and P400-derived polypeptides, as described herein, for inhibition of amyloid formation, deposition, accumulation, and/or persistence, regardless of its clinical setting.

Yet another object of the present invention is to provide compositions and methods involving administering to a subject a therapeutic dose of P400, P400-derived protein fragments, and P400-derived polypeptides, which inhibit amyloid deposition, and fragments thereof. Accordingly, the compositions and methods of the invention are useful for inhibiting amyloidosis in disorders in which amyloid deposition occurs. The proteins or polypeptides of the invention can be used therapeutically to treat amyloidosis or can be used prophylactically in a subject susceptible to amyloidosis. The methods of the invention are based, at least in part, in directly inhibiting amyloid fibril formation, and/or causing dissolution of preformed amyloid fibrils.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of the invention and are not meant to limit the scope of the invention.

FIGURE 1 is a black and white photograph of a ligand blot analysis of serum separated by non-reducing SDS-PAGE, transferred to PVDF membrane, and probed with 5nM of biotinylated-Aß (1-40). Laminin and P400 are demonstrated to be major Aß-binding proteins in human serum. Low concentrations of Aß (1-40) detected only those proteins that have dissociation constants in the nanomolar range. A prominent and more focused ~130 kDa Aß-binding protein (lower arrow) is believed to represent the E8 fragment of laminin (Begovac et al, J. Cell Biol. 113:637-644, 1991; Yurchenco and Cheng, J. Biol. Chem. 268:17286-17299, 1993), whereas a prominent >205 kDa Aß-binding protein (upper arrow) represents P400 which was confirmed not to be immunologically related to laminin (see Fig 2). Lanes 1 and 4 (normal), lanes 3 and 6 (Alzheimer's disease), lanes 2,5, 7-8 (type II diabetes). Note the reduction of P400 in serum of Alzheimer's disease patients.

FIGURE 2 is a black and white photograph of a Western blot demonstrating that P400 is immunologically distinct from laminin. This is a Western blot of human CSF (30 µl/lane, Lanes 1-6) and serum (10 µl/lane, Lanes 7-14) probed with a laminin antibody. See text for individual lane details. Intact laminin (M_r ~850 kDa) (upper arrow) was present in human CSF (lanes 1-6) but not as prominent in human serum (lanes 7-14). No immunostaining was observed in the region >205 kDa up to the gel interface, (compare to Fig 1), indicating that P400 is immunologically distinct from laminin.

FIGURE 3 is a black and white photograph demonstrating isolation of P400 using Sephacryl S1000 gel filtration chromatography. This is an Aß-ligand blot of various fractions derived from Sephacryl S1000 gel filtration chromatography, where a concentrated sample from 10 ml CSF was applied. The blot was probed with 50 nM of biotinylated Aß (1-40). This demonstrates that the Aß-binding P400 (upper arrow) can be enriched relative to the smaller laminin fragments (lower arrow). This blot shows that P400 is present in human CSF and can be purified from other Aß-binding proteins. Coomassie blue staining of similar blots indicate that very little low molecular weight proteins are present in fractions with K_{av} of 0.5 (not shown), indicating that this method is useful in initial purification of P400.

BEST MODE OF CARRYING OUT THE INVENTION

Turning now to the drawings, the invention will be described in a preferred embodiment by reference to the numerals of the drawing figures wherein like numbers indicate like parts.

Amyloid and Amyloidosis

Amyloid is a generic term referring to a group of diverse, but specific extracellular protein deposits which all have common morphological properties, staining characteristics, and x-ray diffraction spectra. Regardless of the nature of the amyloid protein deposited all amyloids have the following characteristics: 1) an amorphous appearance at the light microscopic level and appear eosinophilic using hematoxylin and eosin stains; 2) all stain with Congo red and demonstrate a red/green birefringence as viewed under polarized light (Puchtler et al., J. Histochem. Cytochem. 10:355-364, 1962), 3) all contain a predominant beta-pleated sheet secondary structure, and 4) ultrastructurally amyloid usually consist of non-branching fibrils of indefinite length and with a diameter of 7-10 nm.

Amyloid today is classified according to the specific amyloid protein deposited. The amyloid diseases include, but are not limited to, the amyloid associated with Alzheimer's disease, Down's syndrome and Hereditary cerebral hemorrhage with amyloidosis of the Dutch type (wherein the specific amyloid is referred to as beta-amyloid protein or Aß), the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid is referred to as amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru and animal scrapie (wherein the specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as beta2-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as prealbumin or transthyretin amyloid), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred to as variants of procalcitonin).

Although amyloid deposits in clinical conditions share common physical properties relating to the presence of a beta-pleated sheet conformation, it is now clear that many different chemical types exist and additional ones are likely to be described in the future. It is currently thought that there are several common pathogenetic mechanisms that may be operating in amyloidosis in general. In many cases, a circulating precursor protein may result from overproduction of either intact or aberrant molecules (ex. plasma cell dyscrasias), reduced degradation or excretion (serum amyloid A in some secondary amyloid syndromes and beta2-microglobulin in long-term hemodialysis), or genetic abnormalities associated with

variant proteins (ex. familial amyloidotic polyneuropathy). Proteolysis of a larger protein precursor molecule occurs in many types of amyloidosis, resulting in the production of lower molecular weight fragments that polymerize and assume a beta-pleated sheet conformation as tissue deposits, usually in an extracellular location. What are the precise mechanisms involved, and the aberrant causes leading to changes in proteolytic processing and/or translational modifications is not known in most amyloids.

Systemic amyloids which include the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (ie. AA amyloid or inflammation-associated amyloidosis)(Benson and Cohen, Arth. Rheum. 22:36-42, 1979; Kamei et al, Acta Path. Jpn. 32:123-133, 1982; McAdam et al, Lancet 2:572-573, 1975; Metaxas, Kidney Int. 20:676-685, 1981), and the amyloid associated with multiple myeloma and other B-cell dyscrasias (ie. AL amyloid)(Harada et al, J. Histochem. Cytochem. 19:1 15, 1971), as examples, are known to involve amyloid deposition in a variety of different organs and tissues generally lying outside the central nervous system. Amyloid deposition in these diseases may occur, for example, in liver, heart, spleen, gastrointestinal tract, kidney, skin, and/or lungs (Johnson et al, N. Engl. J. Med. 321:513-518, 1989). For most of these amyloidoses, there is no apparent cure or effective treatment and the consequences of amyloid deposition can be detrimental to the patient. For example, amyloid deposition in kidney may lead to renal failure, whereas amyloid deposition in heart may lead to heart failure. For these patients, amyloid accumulation in systemic organs leads to eventual death generally within 3-5 years. Other amyloidoses may affect a single organ or tissue such as observed with the Aß amyloid deposits found in the brains of patients with Alzheimer's disease and Down's syndrome: the PrP amyloid deposits found in the brains of patients with Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, and kuru; the islet amyloid (amylin) deposits found in the islets of Langerhans in the pancreas of 90% of patients with type II diabetes (Johnson et al, N. Engl. J. Med. 321:513-518, 1989; Lab. Invest. 66:522 535, 1992); the beta2-microglobulin amyloid deposits in the medial nerve leading to carpal tunnel syndrome as observed in patients undergoing long-term hemodialysis (Geyjo et al, Biochem. Biophys. Res. Comm. 129:701-706, 1985; Kidney Int. 30:385-390, 1986); the prealbumin/ transthyretin amyloid observed in the hearts of patients with senile cardiac amyloid; and the prealbumin/ transthyretin amyloid observed in peripheral nerves of patients who have Familial Amyloidotic Polyneuropathy (Skinner and Cohen, Biochem. Biophys. Res. Comm. 99:1326-1332, 1981; Saraiva et al, J. Lab. Clin. Med. 102:590-603, 1983; J. Clin. Invest. 74:104-119, 1984; Tawara et al, J. Lab. Clin. Med. 98:811-822, 1989).

Alzheimer's Disease and the Aging Population

Alzheimer's disease is a leading cause of dementia in the elderly, affecting 5-10% of the population over the age of 65 years (A Guide to Understanding Alzheimer's Disease and Related Disorders, edited by Jorm. New York University Press, New York, 1987). In Alzheimer's disease, the parts of the brain essential for cognitive processes such as memory, attention, language, and reasoning degenerate, robbing victims of much that makes us human, including independence. In some inherited forms of Alzheimer's disease, onset is in middle age, but more commonly, symptoms appear from the mid-60's onward. Alzheimer's disease today affects 4-5 million Americans, with slightly more than half of these people receiving care at home, while the others are in many different health care institutions. The prevalence of Alzheimer's disease and other dementias doubles every 5 years beyond the age of 65, and recent studies indicate that nearly 50% of all people age 85 and older have symptoms of Alzheimer's disease (1997 Progress Report on Alzheimer's Disease, National Institute on Aging/National Institute of Health). 13% (33 million people) of the total population of the United States are age 65 and older, and this % will climb to 20% by the year 2025 (1997 Progress Report on Alzheimer's Disease, National Institute on Aging/National Institute of Health).

Alzheimer's disease also puts a heavy economic burden on society as well. A recent study estimated that the cost of caring for one Alzheimer's disease patient with severe cognitive impairments at home or in a nursing home, is more than $47,000 per year (A Guide to Understanding Alzheimer's Disease and Related Disorders, edited by Jorm, New York University Press, New York, 1987). For a disease that can span from 2 to 20 years, the overall cost of Alzheimer's disease to families and to society is staggering. The annual economic toll of Alzheimer's disease in the United States in terms of health care expenses and lost wages of both patients and their caregivers is estimated at $80 to $100 billion (1997 Progress Report on Alzheimer's Disease, National Institute on Aging/National Institute of Health).

Tacrine hydrochloride ("Cognex"), the first FDA approved drug for Alzheimer's disease is a acetylcholinesterase inhibitor (Cutler and Sramek, N. Engl. J. Med. 328:808 810, 1993). However, this drug has showed limited success in the cognitive improvement in Alzheimer's disease patients and initially had major side effects such as liver toxicity. The second more recently FDA approved drug, donepezil (also known as "Aricept"), which is also an acetylcholinesterase inhibitor, is more effective than tacrine, by demonstrating slight cognitive improvement in Alzheimer's disease patients (Barner and Gray, Ann. Pharmacotherapy 32:70-77, 1998; Rogers and Friedhoff, Eur. Neuropsych. 8:67-75, 1998), but is not believed to be a cure. Therefore, it is clear that there is a need for more effective treatments for Alzheimer's disease patients.

Amyloid as a Therapeutic Target for Alzheimer's Disease

Alzheimer's disease is characterized by the deposition and accumulation of a 39-43 amino acid peptide termed the beta-amyloid protein, Aß or ß/A4 (Glenner and Wong, Biochem. Biophys. Res. Comm. 120:885-890, 1984; Masters et al, Proc. Natl. Acad. Sci. U.S.A. 82:4245-4249, 1985; Husby et al, Bull WHO 71:105-108, 1993). Aß is derived from larger precursor proteins termed beta-amyloid precursor proteins (or ßPPs) of which there are several alternatively spliced variants. The most abundant forms of the ßPPs include proteins consisting of 695, 751 and 770 amino acids (Tanzi et al, Nature 331:528-530, 1988; Kitaguchi et al, Nature 331:530-532, 1988; Ponte et al, Nature 331:525-527, 1988).
The small Aß peptide is a major component which makes up the amyloid deposits of "plaques" in the brains of patients with Alzheimer's disease. In addition, Alzheimer's disease is characterized by the presence of numerous neurofibrillary "tangles", consisting of paired helical filaments which abnormally accumulate in the neuronal cytoplasm (Grundke-Iqbal et al, Proc. Natl. Acad. Sci. U.S.A. 83:4913-4917, 1986; Kosik et al, Proc. Natl. Acad. Sci. U.S.A. 83:4044-4048, 1986; Lee et al, Science 251:675-678, 1991). The pathological hallmarks of Alzheimer's disease is therefore the presence of "plaques" and "tangles", with amyloid being deposited in the central core of plaques. The other major type of lesion found in the Alzheimer's disease brain is the accumulation of amyloid in the walls of blood vessels, both within the brain parenchyma and in the walls of meningeal vessels which lie outside the brain. The amyloid deposits localized to the walls of blood vessels are referred to as cerebrovascular amyloid or congophilic angiopathy (Mandybur, J. Neuropath. Exp. Neurol. 45:79-90, 1986; Pardridge et al, J. Neurochem. 49:1394-1401, 1987).

For many years there has been an ongoing scientific debate as to the importance of "amyloid" in Alzheimer's disease and whether the "plaques" and "tangles" characteristic of this disease, were a cause or merely the consequences of the disease. Within the last few years, studies now indicate that amyloid is indeed a causative factor for Alzheimer's disease and should not be regarded as merely an innocent bystander. The Alzheimer's Aß protein in cell culture has been shown to cause degeneration of nerve cells within short periods of time (Pike et al, Br. Res. 563:311-314, 1991; J. Neurochem. 64:253-265, 1995). Studies suggest that it is the fibrillar structure (consisting of a predominant ß-pleated sheet secondary structure), characteristic of all amyloids, that is responsible for the neurotoxic effects. Aß has also been found to be neurotoxic in slice cultures of hippocampus (Harrigan et al, Neurobiol. Aging 16:779-789, 1995) and induces nerve cell death in transgenic mice (Games et al, Nature 373:523-527, 1995; Hsiao et al, Science 274:99-102, 1996). Injection of the Alzheimer's Aß into rat brain also causes memory impairment and neuronal dysfunction (Flood et al, Proc. Natl. Acad. Sci. U.S.A. 88:3363-3366, 1991;

Br. Res. 663:271-276. 1994). Probably, the most convincing evidence that Aß amyloid is directly involved in the pathogenesis of Alzheimer's disease comes from genetic studies. It has been discovered that the production of Aß can result from mutations in the gene encoding, its precursor, beta amyloid precursor protein (Van Broeckhoven et al, Science 248:1120-1122, 1990; Murrell et al, Science 254:97-99, 1991; Haass et al, Nature Med. 1:1291-1296. 1995). The identification of mutations in the beta-amyloid precursor protein gene which causes early onset familial Alzheimer's disease is the strongest argument that amyloid is central to the pathogenetic process underlying this disease. Four reported disease-causing mutations have now been discovered which demonstrate the importance of Aß in causing familial Alzheimer's disease (reviewed in Hardy, Nature Genet. 1:233-234, 1992). All of these studies suggest that providing a drug to reduce, eliminate or prevent fibrillar Aß formation, deposition, accumulation and/or persistence in the brains of human patients is believed to serve as an effective therapeutic.

Soluble Beta-Amyloid Protein in Biological Fluids

A number of different studies have now confirmed that Aß is normally present in biological fluids including its presence in both cerebrospinal fluid (CSF)(Shoji et al, Science 258:126-129, 1992; Seubert et al, Nature 359:325-327, 1992; Vigo-Pelfrey et al, J. Neurochem. 61:1965-1968, 1993) and plasma (Seubert et al, Nature 359:325-327, 1992). Seubert et al (Nature 359:325-327, 1992) found Aß peptide in both CSF and plasma of humans, dogs, guinea pigs and rats. In humans, the concentration of Aß in CSF is approximately 2.5 ng/ml whereas in plasma the Aß concentration is approximately 0.9 ng/ml (Seubert et al, Nature 359:325-327, 1992). Although Aß in CSF appears to consist of various lengths, 70% of Aß in CSF consists of Aß 1-34 and Aß 1-40 (Vigo-Pelfrey et al, J. Neurochem. 61:1965-1968, 1993). Since previous studies have demonstrated that synthetic peptides homologous to Aß 1-40 and 1-42 spontaneously form amyloid-like fibrils (Kirschner et al, Proc. Natl. Acad. Sci. U.S.A. 84:6953-6957, 1987; Barrow et al, J. Mol. Biol. 225:1075-10793, 1992), the question arises as to why Aß in biological fluids is maintained in a soluble state? It is currently believed that CSF and plasma/serum contain additional factors that maintain Aß in a soluble state preventing its potential fibrillogenesis.

Previous studies (Wisniewski et al, Biochem. Biophys. Res. Commun. 192:359-365, 1993) indicated that some components of CSF are able to inhibit fibril formation and aggregation of Aß peptide such that it remains soluble. Apolipoproteins E and J (in blood and CSF with approximate SDS-PAGE mobility of 80 kDa or less) (Ghiso et al, Biochem. J. 293:27-30, 1993; Wisniewski et al, Biochem. Biophys. Res. Commun. 192:359-365, 1993; Strittmatter et al, Proc. Natl. Acad. Sci. U. S. A. 90:8098-8102, 1993; LaDu et al, J. Biol. Chem. 269:23403-23406, 1994; Naslund et al, Neuron 15:219-228, 1995; Chan et al, Biochemistry 35:7123-7130, 1996), and albumin (in blood) (Biere et al, J. Biol. Chem.

271:32916-32922. 1996) are proteins previously implicated in keeping Aß in a soluble state in biological fluids. Previous studies have also demonstrated that apoproteins bind Aß within the µM range (Strittmatter et al, Proc. Natl. Acad. Sci. U. S. A. 90:8098-8102, 1993; LaDu et al, J. Biol. Chem. 269:23403-23406. 1994) and their ability to bind Aß is further attenuated when complexed with lipids (Biere et al, J. Biol. Chem. 271:32916-32922. 1996). In our previous studies (Castillo and Snow, unpublished data) using a solid phase binding assay. we determined that albumin binds Aß with a dissociation constant of 0.05 mM, which is suggestive of rather weak binding. However, due to the normally high concentrations of albumin in blood (1 mM), this binding appears significant if one increases the concentration of Aß, by adding exogenous Aß to the blood samples. as was employed in a previous study (Biere et al. J. Biol. Chem. 271:32916-32922, 1996). In the CSF however, the albumin concentration is only 3 µM, which makes the binding of albumin to Aß with a K_d of 0.05 mM, insignificant in this not very tight biological fluid compartment. Our previous studies have demonstrated that laminin in serum and CSF binds Aß at low nanomolar concentrations (with a K_d = 2.7 x 10-9 M), suggesting that laminin (and/or laminin fragments) in blood and CSF can also contribute to Aß solubility (Castillo et al, Soc. Neurosci. Abst. 23:1882, 1997).

However, each of the described proteins above may only be partially responsible for maintenance of Aß in a soluble state in biological fluids. Identification of other proteins which are also present in biological fluids and which bind Aß very tightly, may also serve to maintain Aß in a soluble state. Data presented in this invention indicates that we have detected a new unidentified protein in serum and CSF which binds Aß much tighter that any of the previous proteins implicated in biological fluids to maintain Aß in a soluble state (i.e. lipoproteins, albumin and laminin).

Examples

The following examples are put forth so as to provide those with ordinary skill in the art with the disclosure and description of the discovery of P400 as a new Aß-binding protein in serum and CSF, and its use as a diagnostic marker for Alzheimer's disease and/or its progression.

Example 1

Identification of a ~350-450 Kilodalton Protein in Human Serum which Binds Aß and is Reduced in Alzheimer's Disease

Aß ligand blotting techniques were utilized to identify proteins present in human serum which bind Aß. In this initial study, human serum was obtained from normal aged and Alzheimer's disease living patients who mostly have had corresponding mini-mental state examinations (where a score of 30 is normal, a score of 15 suggests moderate dementia, and a score <10 suggests severe dementia), or from patients with confirmed type II diabetes. For ligand blot analysis, 1µl of human serum was separated by non-reducing SDS-PAGE, transferred to PVDF membrane and probed with 5 nM of biotinylated Aß 1-40. The probing with low concentrations of Aß 1-40 will detect only those proteins which had a dissociation constant in the nanomolar range. In Fig. 1, the following human serums were obtained and analyzed as part of this study: Lane 1: patient #9; a normal 67 yr. old female with a mini-mental score of 30; Lane 2: patient B- a 63 year old male with confirmed type II diabetes; Lane 3: patient #5223- a 68 year old female with probable Alzheimer's disease who had a mini-mental score of 22; Lane 4: patient #22- an 83 yr. old normal aged female who had a mini-mental score of 30; Lane 5: patient #E- a 54 year old male with confirmed type II diabetes; Lane 6: patient #5230- a 72 year old female with probable moderate Alzheimer's disease who had a mini-mental score of 19; Lane 7: patient #E-a 54 year old male with confirmed type II diabetes; and Lane 8: patient #F- a 69 year old male with confirmed type II diabetes.

A prominent ~130 kDa Aß-binding band (lower arrow) was present in all patients serums and was believed to represent the E8 fragment of laminin (Yurchenco et al, J. Biol. Chem. 268:17286-17299, 1993). A prominent >205 kilodalton band (and designated as P400 due to an estimated approximate size of ~350-450 kilodaltons)(upper arrow) is also present in human serum derived from normal (lanes 1 and 4) and type II diabetes (lanes 2, 5, 7 and 8) patients, and appears to be markedly decreased in the serum derived from 2 patients with moderate AD (lanes 3 and 6). All of these serums were similarly obtained from live patients so the decreased P400 in the serum of probable AD patients is not believed to represent "breakdown" products. Evidence also indicates that P400 is also present in human CSF samples (see Fig. 3).

Example 2

P400 is Not Immunologically Related to Laminin

In the next study, a polyclonal antibody against laminin was used to probe (by Western blot) human CSF and serum samples to determine whether P400 was immunologically related to laminin. In this study, human CSF (30 µl/lane, lanes 1-6) and serum (10 µl/lane, lanes 7-14) was probed with a polyclonal antibody against laminin (obtained from Sigma Chemical Co., St. Louis. MO). In Figure 2, the

following human biological fluid samples (i.e. CSF and serum) were obtained and analyzed as part of this study: Lane 1: #5211- CSF from a 66 year old male with probable AD with a mini-mental score of 25; Lane 2: #5113- CSF from a 83 year old normal male with a mini-mental score of 27; Lane 3: #5112- CSF from a 78 year old normal female with a mini-mental score of 30; Lane 4: #5111- CSF from a 69 year old female with presymptomatic Alzheimer's disease with mini-mental score of 28; Lane 5: #5110- CSF from a 67 year old normal male with a mini-mental score of 30; Lane 6: #5109- CSF from a 78 year old normal female with a mini-mental score of 30. The corresponding serum from these same patients are in Lanes 7-12, and in the same order as the CSF samples. Lane 13: #5101- serum from a 73 year old normal male with a mini-mental score of 30; Lane 14: #5216- serum from a 74 year old female with confirmed Alzheimer's disease who had a mini-mental score of 14.

As shown in Fig. 2, intact laminin (M_r ~850 kilodaltons)(which is observed at the gel interface and does not enter the gel due to its large size) (upper arrow) was present in human CSF (lanes 1-6) and to a lessor extent in human serum (lanes 7-14). The broad band observed between the 116 and 205 kDa markers is believed to represent the E8 fragment of laminin (Begovac et al, Cell Biol. 113:637-644, 1991; Yurchenco et al, J. Biol. Chem. 268:17286-17299, 1993). The diffuseness of this band (in comparison to Fig. 1) is believed to be due to the use of a polyclonal antibody against laminin which will recognize multiple epitopes. The faintness of this band is believed to be due to the presence of the E8 fragment of laminin at low levels in CSF and serum. No immunostaining for laminin was observed in the region >205 kilodaltons (up to the gel interface) suggesting that P400 was likely not immunologically related to laminin (compare the region >205 kilodaltons up to the gel interface in Fig. 1 versus Fig. 2). We have employed other antibodies against laminin and have observed identical results (not shown), suggesting that P400 is not immunologically related to laminin.

Example 3

Isolation and Purification of P400 from Human Cerebrospinal Fluid

Data also indicated that P400 is present in human CSF and can be isolated by Sephacryl S1000 fractionation (Fig. 3). For these studies 10 ml of CSF were fractionated through an Sephacryl S1000 column, and the various fractions were separated by non-reducing SDS-PAGE, transferred to PVDF membrane and probed with 50 nM of biotinylated-Aß. This concentration of biotinylated-Aß was chosen so as to detect most proteins in CSF which bind Aß. As shown in Fig. 3, P400 (upper arrow) is present in human CSF and can be enriched using Sephacryl S1000 fractionation. The Aß-ligand blot (Fig. 3) demonstrated that P400 is present in human CSF and can be purified from other Aß-binding proteins. Coomassie blue staining of similar blots indicate that very little low molecular weight proteins were

present in fractions with K_{av} of 0.5 (not shown), indicating that this method is useful in initial purification of P400.

Example 4

Methodology to Determine the Identity of P400

Rationale: Our initial evidence suggests that P400 is present in both serum and CSF in humans, binds Aß, and may be a potential marker for AD. Gel filtration chromatography and Aß-immunoaffinity chromatography are methods used to isolate P400 in sufficient quantities for microsequencing. Comparisons are then be made to proteins in known data bases to identify P400.

Methodology: Approximately 30 mls of serum from each of pooled Alzheimer's disease patients and normal aged controls are used. For this study, 30 mls of serum (from each of Alzheimer's disease and normal controls) is lyophilized separately and fractionated on a 40 ml Sephacryl S-1000 equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 1 M urea (pH 8.0), followed by elution using the same buffer. Our preliminary analysis (Fig. 3) indicates that P400 is enriched in the fractions with a K_{av} = 0.45-0.55. Therefore, for this purification of P400, fractions with K_{av} = 0.45-0.55 are collected and precipitated by centrifugation at 14,000 Xg in the presence of 4 volumes of 95 % ethanol with 1 % sodium acetate. The precipitate obtained is then dissolved in 2 ml of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS) and passed through an Aß affinity column for further purification.

For preparation of the Aß affinity column, 1 mg of Aß 1-40 (Bachem Inc, Torrance, CA) in 0.5 ml of double distilled water is added to 1 ml of washed Affigel beads (Biorad) suspended in 1 volume of 0.2 M HEPES 160 mM CaCl (pH 7.5). The mixture is incubated overnight with gentle agitation at room temperature and stopped with 100µl of 1 M ethanolamine at pH 8.0 for 1-2 hours at room temperature. The derivatized gel is washed in a minicolumn and the flow through is collected and assayed for protein content using a Buffalo black or Bradford reagent (Biorad) to determine the % of protein bound to the column. Previous studies (Snow et al, Arch. Biochem. Biophys. 320:84-95, 1995) indicated that about 95 % of Aß can be coupled to the column using this protocol. The column is first be equilibrated with TBS containing 0.1 % Triton X-100. Then, P400 (isolated as described above) is dissolved in TBS and is repeatedly applied to the column four times. Unbound materials are washed with 10 ml TBS containing 0.1 % Triton X-100. The bound materials are eluted with a 40 ml-linear gradient of NaCl (0.15-1.0 M) in TBS containing 0.1 % Triton X-100 at pH 7.5. Tightly bound materials are then be eluted with 10 ml of 50 mM Tris-HCl, 3M NaCl, 7 M Urea (pH 8.0). Aliquots of each of 2ml fractions are precipitated by

15-minute centrifugation at 14,000 Xg in the presence of 4 volumes of 95 % ethanol with 1 % sodium acetate. The pellets are then made up to 10 µl 1 x SDS sample buffer and are subjected to SDS-PAGE and transferred to PVDF membrane (see below) to identify fractions containing P400. Identification of P400 is based on size and its ability to bind Aß as determined by Aß ligand blot analysis.

SDS-PAGE (4-15 % Tris-Glycine precast gels from BioRad, Hercules, CA) is performed under non-reducing conditions according to the method of Laemmli (Nature 227:680-685, 1970) using a Mini-Protean II electrophoresis system (BioRad). All samples are in 1 X SDS sample buffer and heated for 5 minutes in a boiling water bath before electrophoresis at 200V for 45 minutes along with pre-stained molecular weight protein standards. The separated proteins are transferred from the gel to PVDF membrane (Immobilon-P for Western and ligand blot or Immobilon-PSQ for sequencing, Millipore, Bedford, MA) using a Mini-transblot electrophoresis transfer cell according to manufacturers protocol (BioRad). Electrotransfer is performed at 100 V for 2 hours. Following transfer, membranes are rinsed with methanol and dried.

For Coomassie blue staining, PVDF membranes are immersed with 0.2 % Coomassie brilliant blue (w/v) in 50 % methanol, 10 % acetic acid, and 40% distilled water for 2 minutes. The membranes are then rinsed with 50% methanol, 10% acetic acid, and 40% distilled water until the bands are visible and no background staining is remaining. The corresponding Aß-binding P400 band (determined by ligand blotting of adjacent lanes as described below) is sent out for amino-acid sequencing. Amino acid sequencing is conducted using a Porton 2090 Gas-Phase Microsequencer with on-line analysis of phenylthiohydantoin derivatives (Porton Instruments. Tarzana. CA) as previously described (Castillo and Templeton, FEBS Lett. 318:292-296, 1993). If the protein has a blocked N-terminus, protease digestions are performed to expose internal portions of the protein, which are anticipated to allow for N-terminal sequencing. For these protease digestion studies, P400 is either left undigested, or digested with V8, trypsin, or elastase (Sigma Chem. Co., St. Louis, MO) prior to SDS-PAGE. More specifically, 2 µg of trypsin, V8 protease, or elastase in 2 µl of 50 mM Tris-HCl buffer (pH 8.0) is added to either buffer only, or to 50 µl of P400 (50 µg) in the same buffer. The mixtures are incubated at 37°C and aliquots (10 µl) taken at various times are mixed with equal volumes of 2X non-reducing SDS-sample buffer for SDS-PAGE. The separated P400 fragments are then transferred to PVDF membranes and visualized by staining with Coomassie blue as described above. The most abundant P400 fragments as judged by Coomassie blue staining intensity are also be sent for microsequencing. The partial P400 sequences obtained by microsequencing is then be identified by comparison to known protein sequence libraries retrieved from the National Center for Biotechnology Information, Bethesda, MD. Initial studies indicate that 20-40 ml of

serum gives a final yield of 0.4-0.8 mg of P400, which is more than sufficient for incorporation into all of the studies described.

A more accurate molecular weight of P400 is also determined by slightly decreasing the acrylamide concentration (from 4% to 3.5%) and include larger molecular weight standards (i.e. >400 kDa) obtained commercially.

Example 5

Determination of the Strength of Binding of P400 to Beta-Amyloid Protein

Rationale: The data indicates that P400 binds to Aß with high affinity in human biological fluids. In this study, solid phase binding assays are used to determine the dissociation constant of P400 to immobilized Aß using biotinylated-P400. As an alternative assay to P400-biotinylation, polyclonal antibodies to P400 are generated in rabbits and used in ELISA assays to determine the dissociation constant of Aß binding to P400 as previously described (Castillo et al, J. Neurochem. 69:2452-2465, 1997).

Methodology: To biotinylate P400, 0.5 mg of purified P400 is diluted into 100 µl PBS, transferred into a tube containing 0.10 mg Sulfo-NHS-LC-Biotin, and incubated for 45 minutes at room temperature. Biotinylated-P400 is then precipitated by addition of 95 % ethanol containing 1 % sodium acetate (w/v) followed by centrifugation at 14,000 Xg for 20 minutes. The supernatant is decanted and P400 is rinsed twice by re-precipitation as above. Biotin incorporation is determined per molecule of P400 using [2-(4'-hydroxyazobenzene)benzoic acid] (HABA) according to the manufacturer's protocol (Pierce, Rockford, Il).

For solid phase binding assays, Nunc plates (Maxisorb) containing immobilized Aß is first prepared by incubating each well overnight with 2 µg of Aß (Bachem Inc, Torrance, CA) in 40 µl of TBS containing 100mM Tris-HCl, 50 mM NaCl, and 3 mM NaN3 (pH 7.4). The next day the wells are blocked with 300 µl of TBS containing 0.05% Tween-20 (TTBS) plus 2% bovine serum albumin (BSA). Wells without immobilized Aß (i.e. blank wells) are also blocked with TTBS containing 2 % albumin as described above. Purified and biotinylated P400 (1 mg/ml) is diluted in TTBS at dilutions of 1:10, 1:30, 1:90, 1:270, 1:810, 1:2430, 1:7290 (v/v) and blank. These dilutions are plated (250 µl) in triplicate wells in the presence or absence of immobilized Aß (1-40) and incubated overnight. The next day, the wells are rinsed 3 times with TTBS and probed for 30 min with 100 µl of streptavidin-peroxidase (1:500 of 2 µg/ml) in TTBS containing 0.1 % BSA (i.e. secondary probe). The wells are then rinsed 3 times with TTBS and 100 µl of substrate solution (OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO.) is added to each well and allowed to develop for 10 minutes or until there is a significant color difference.

The reaction is stopped with 50 µl of 4 N sulfuric acid and read at 490 nm on a Model 450 ELISA plate reader (Biorad). The data is plotted and the K_d is determined using the Ultrafit program (Biosoft, UK).

If there is a problem with proper biotinylation of P400 (for example, biotinylation of P400 may block the binding site on P400 which normally associates with Aß; this would be determined by comparing K_d's obtained using ELISA techniques to the described ligand studies), then an alternative method as described below can be used. For this method, rabbit polyclonal antibodies are utilized to detect the relative amount of P400 bound to Aß immobilized on microtiter wells. Briefly, P400 at various dilutions described above are plated (250 µl) in triplicate wells in the presence or absence of immobilized Aß (1-40) and incubated overnight. The next day the wells are rinsed 3 times with TTBS and probed for 1 hour with 100 µl of an anti-P400 polyclonal antibody diluted in TTBS (dilution to be determined empirically). After 3 rinses with TTBS, the wells are incubated for 30 min with 100 µl of biotinylated goat anti-rabbit secondary antibody (1:1000), and streptavidin-peroxidase (1:500 of 2 µg/ml) in TTBS containing 0.1 % BSA. The wells are then be rinsed 3 times with TTBS and 100 µl of the substrate solution (OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO.) is then added to each well and allowed to develop for 10 minutes or until there is a significant color difference. The reaction is stopped with 50 µl of 4 N Sulfuric acid and read at 490 nm on an ELISA plate reader. The data is plotted and the K_d is determined using the Ultrafit program (Biosoft, UK).

The binding data is analyzed assuming a thermodynamic equilibrium for the formation of the complex BL, from the P400 ligand in solution, L, and the uncomplexed Aß adsorbed to the microtiter well, B, according to the equation: $K_d = [B] \times [L]/[BL]$ (Castillo et al, J. Neurochem. 69:2452-2465, 1997). The K_d's are determined by using an enzyme-linked immunoassay that gives a color signal that is proportional to the amount of P400 bound to Aß (Engel and Schalch, Mol. Immunol. 17:675-680, 1980; Mann et al, Eur. J. Biochem. 178:71-80, 1988; Fox et al, EMBO J. 10:3137-3146, 1991; Battaglia et al, Eur. J. Biochem. 208:359-366, 1992). To account for potential non-specific binding, control wells without Aß (in triplicate) are included for each concentration of P400 used in each binding experiment. Optical densities of the control wells usually never exceed 0.050 at all ligand concentrations based on previous studies (Castillo et al, J. Neurochem. 69:2452-2465, 1997). The optical densities of the control wells are subtracted from the optical densities of the Aß-containing wells that received similar P400 concentrations. Non-specific absorbance obtained from Aß containing wells that did not receive P400 are also subtracted from all data points. Thus, the equation in the form of: $OD_{exp} = OD_o + (S \times [P400]) + (OD_{max} \times [P400]/([P400] + K_d)$ where $(S \times [P400])$ represents non-specific binding (control wells) and OD_o is the non-specific absorbance, becomes $OD_{exp} = OD_{max} \times [P400]/([P400] + K_d)$. Therefore, at 50 % saturation

$OD_{exp} = 0.50\ OD_{max}$ and K_d = [P400]. Determination of [P400] at 50% saturation is performed by non-linear least square program (Ultrafit from Biosoft, UK) using a one-site model.

For preparation of polyclonal antibodies against P400, Zymed Laboratories are used. Once pure P400 is isolated, 100-200 µg are sent out to Zymed labs for the preparation of polyclonal antibodies. If necessary, affinity purification of P400 antibodies is performed, once the initial polyclonal antibodies have arrived. For such a procedure, 0.5 mg of P400 is incubated with 1 ml of a washed Affigel bead (Biorad) suspension in 0.5 ml 0.2 M HEPES 160 mM CaCl (pH 7). The reaction is stopped with 100µl of 1 M ethanolamine at pH 8.0 for 1-2 hours at room temperature. The derivatized gel is washed in a minicolumn and the flow-through is collected and assayed for protein content using the Bradford reagent according to manufacturer protocol (Biorad). The column is equilibrated with TBS with 0.1 % TX-100 and 10 ml of serum diluted 1:4 in 100 mM Tris-HCl, 50 mM NaCl, pH 7.5 (TBS) is applied several times. Unbound materials are washed with 20 ml TBS. Bound antibodies are eluted with 1 % acetic acid/water and lyophilized.

Example 6

Effects of P400 on Inhibition/Dissolution of Aß Fibril Formation

Rationale: The initial data demonstrates that P400 binds to Aß with high affinity in biological fluids. P400 is believed to be a potent inhibitor of Aß fibril formation and likely dissolves pre-formed Aß amyloid fibrils. For these studies, Thioflavin T fluorometry, Congo red staining assays and negative stain electron microscopy are employed to determine the effects of purified P400 on inhibition of Aß fibril formation and whether P400 is capable of dissolving preformed Aß amyloid fibrils.

Methodology: To assess the potential inhibitory effects of P400 on Aß amyloid fibril formation, fluorometry assays will be used as previously described (Castillo et al, J. Neurochem. 69:2452-2465, 1997). Briefly, 25 µM of Aß (1-40) (Bachem Inc., Torrance, CA; Lot # WM 365) in TBS solution (pH 7.0) is incubated at 37°C (in triplicate) in the presence or absence of 100 nM of P400. The mixtures are incubated for 1 week at 37°C with aliquots analyzed at 1h, 1 day, 3 days and 1 week. Fibrillogenesis is determined by addition of 1.2 ml Thioflavin T solution (100 µM Thioflavin T in 50 mM Sodium phosphate buffer pH 6.0) to 50 µl aliquots, followed by reading on the fluorometer as previously described (Castillo et al, J. Neurochem. 69:2452-2465, 1997). The effect of various concentrations of P400 (5-100nM) on Aß fibrillogenesis (25 µM) is also tested in a similar manner as described above. The effect of various concentrations of P400 (5-100nM) on another amyloid protein called amylin (25 µM; Bachem Inc.)(which accumulates in the pancreas of 90% of patients with type II diabetes) is also determined to

give an idea of amyloid specificity. Previous studies (Castillo et al, J. Neurochem. 69:2452-2465, 1997) indicate that increasing concentration of fibrillar Aß in this assay gives a proportional increase in fluorescence in the presence of 100 µM Thioflavin T, ruling out the presence of any disproportionate inner filter effects in these studies. The increase in Thioflavin T fluorescence with time is proportional to the increase in the amount of amyloid fibrils with time. Initial data indicates that P400 does not contribute to Thioflavin T fluorescence when measured alone:

In another fluorometry experiment, several different concentrations of P400 are used to determine the dose-dependent effects of P400 on dissolution of pre-formed Aß amyloid fibrils. For this fibril dissolution assay, 250 µM of Aß (1-40) are allowed to fibrillize for 1 week following incubation at 37°C. In addition, 250 µM of Aß 1-42 (which does not require pre-fibrillation) is used. Aliquots are then made up to 25 µM with various concentrations of P400 and incubated for 4 days at 37°C, and analyzed using Thioflavin T fluorometry assays as described above. 5-10 µl aliquots from various incubation mixtures are also be spotted on gelatin-coated glass slides and air-dried overnight, before staining with Congo red the next day (Puchtler et al, J. Histochem. Cytochem. 10:355-364, 1962). Whether a postulated decrease in fibril formation correlates with a decrease in Congo red staining is determined. P400 potential inhibition of Aß amyloid fibril formation as determined by Thioflavin T fluorometry and Congo red staining assays are confirmed by negative stain electron microscopy. For these studies, negatively stained fibrils are prepared by floating pioloform, carbon-coated grids on peptide solutions (1 to 2 mg/ml) in the presence or absence of increasing concentrations of P400 (to be determined empirically). To control for pH changes, peptides are dissolved in buffered solutions of 20 mM glycine (for pH 2 to 3 and pH 9 to 10) or 20 mM Tris -HCL (for pH 6 to 8). After the grids are blotted and air-dried, the samples are stained with either 2% (w/v) uranyl acetate or 1% (w/v) phosphotungstic acid and visualized, and photographed with a JEM 1200 EX II (JEOL Ltd, Tokyo, Japan), using 80kV accelerating voltage.

Example 7

P400 as a Specific Diagnostic Marker For Alzheimer's Disease

Rationale: The data suggests that P400 is reduced in the biological fluids in Alzheimer's disease and may therefore potentially serve as a specific biochemical marker. Studies utilizing Aß-ligand blotting techniques followed by scanning densitometry can quantitate changes in relative intensity in P400 among Alzheimer's disease versus normal aged-matched control patients. In addition, sandwich or competitive ELISAs are used to determine whether P400 is useful as a specific marker protein for Alzheimer's disease and/or its progression.

Methodology: Aß-ligand blotting techniques are used to assess levels of P400 in biological fluids of Alzheimer's disease versus normal aged-matched controls. Biotinylation of Aß 1-40, and assessment of serum and CSF samples by Aß-ligand blotting is as described in example 5. Following transfer of serum and CSF proteins to PVDF membranes as described in example 5, membrane blots are probed for 2 hrs with 50 nM biotinylated Aß (1-40) in TTBS which is heated to 90°C for 5 minutes prior to use. The membranes are then rinsed three times (5 seconds each) with TTBS, probed for 30 minutes with streptavidin alkaline phosphatase conjugate (Vectastain), rinsed again (as described above), followed by the addition of an alkaline phosphatase substrate solution (Vectastain). Following color development, flushing the membranes with distilled deionized water stops the reaction. The relative intensity of P400 bands is determined by scanning the blots and quatifying using a Biorad scanning densitometer, as previously described (Maresh et al, Soc. Neurosci. Abst. 23:2221, 1997).

If specific polyclonal antibodies against P400 are obtained as described in example 5, then a sandwich ELISA to quantitate levels of P400 in serum and CSF from Alzheimer's disease versus normal aged patients is employed. For these studies, Aß is immobilized on the plate and serves as the capture molecule. CSF or serum is then applied, and any P400 bound to Aß is detected using the anti-P400 antibody. More specifically, Nunc plates (Maxisorb) containing immobilized Aß 1-40 or 1-42 (Bachem Inc., Torrance CA) is prepared by incubating each well overnight with 2 µg of Aß in 40 µl Tris-buffered saline (TBS; 100 mM Tris-HCl, 50 mM NaCl, and 3 mM NaN3, pH 7.4) and blocking the next day with 300 µl TBS containing 0.05% Tween-20 (TTBS) plus 2% bovine serum albumin (BSA). Wells without immobilized Aß are also blocked with TTBS containing 2 % albumin as described above (i.e. blank wells). Serum samples diluted 1:100 are applied in triplicate wells in the presence or absence of immobilized Aß (1-40), and then incubated overnight. Known amounts of purified P400 are also applied to serve as an internal quantitative standard. The next day, the wells are rinsed 3 times with TTBS and probed for 30 min with 100 µl of a secondary probe consisting of peroxidase conjugated anti-rabbit antibody diluted 1:500 (out of 2 µg/ml)(Jackson immunoresearch, Westgrove, PA) in TTBS containing 0.1 % BSA. The wells are then rinsed 3 times with TTBS, and 100 µl of substrate solution (OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO.) is added to each well and allowed to develop for 10 minutes or until there is a significant color difference. The reaction is stopped with 50 µl of 4 N Sulfuric acid and read at 490 nm on an ELISA plate reader. The optical density of various concentrations of standard P400 is plotted, and the concentrations of P400 in CSF and serum samples is determined based on the standard curve.

If polyclonal antibodies against P400 are not available, levels of P400 in biological fluids are quantitated by a competitive ELISA format. The competitive ELISA is performed based on the competition for binding of biotinylated P400 versus nonbiotinylated P400 (in serum or CSF) to immobilized Aß. In this scenario, the amounts of biotinylated P400 bound to immobilized Aß is inversely proportional to the amounts of P400 in the biological sample. More specifically, Nunc plates (Maxisorb) containing immobilized Aß (1-40 or 1-42; Bachem Inc., Torrance, CA) is prepared by incubating each well overnight with 2 µg of Aß in 40 µl Tris-buffered saline (TBS; 100 mM Tris-HCl, 50 mM NaCl, and 3 mM NaN3, pH 7.4) and blocking the next day with 300 µl TBS containing 0.05% Tween-20 (TTBS) plus 2% bovine serum albumin (BSA). Wells without immobilized Aß are also blocked with TTBS containing 2 % albumin as described above (i.e. blank wells). Various known standard concentrations of nonbiotinylated P400 and serum or CSF samples with unknown concentrations of P400 are mixed with fixed concentration of biotinylated-P400. The fixed concentration of biotinylated-P400 is chosen so that it is close to saturating all of the immobilized Aß sites in each well. These solutions are applied in triplicate wells in the presence or absence of immobilized Aß (1-40) and incubated overnight. The next day the wells are rinsed 3 times with TTBS and probed for 30 min with 100 µl of secondary probe made up with streptavidin-peroxidase (1:500 of 2 µg/ml) in TTBS containing 0.1 % BSA. The wells are then rinsed 3 times with TTBS and 100 µl of the substrate solution (OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO.) is added to each well and allowed to develop for 10 minutes or until there is a significant color differences. The reaction is stopped with 50 µl of 4 N Sulfuric acid and read at 490 nm. A standard curve is then plotted from the optical density results of the standard concentrations of P400, which is an inverse relationship. The concentrations of P400 in CSF and serum is determined based on the standard curve.

Further Aspects and Utilizations of the Invention

P400-Derived Protein Fragments and Polypeptides

One therapeutic application of the present invention is to use P400, P400 protein fragments which bind Aß or other amyloid proteins, and/or P400 polypeptides derived from amino acid sequencing of the P400 fragments which bind Aß or other amyloid proteins, as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses. The amyloid diseases include, but are not limited to, the amyloid associated with Alzheimer's disease and Down's syndrome (wherein the specific amyloid is referred to as beta-amyloid protein or Aß), the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever

(wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid is referred to as amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann Straussler syndrome, kuru and animal scrapie (wherein the specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as beta2-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as transthyretin or prealbumin), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred to as variants of procalcitonin).

The polypeptides referred to above may be a natural polypeptide, a synthetic polypeptide or a recombinant polypeptide. The fragments, derivatives or analogs of the polypeptides to any P400 fragment referred to herein may be a) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be encoded by the genetic code, or b) one in which one or more of the amino acid residues includes a substituent group, or c) one in which the mature polypeptide is fused with another compound, such as a compound used to increase the half-life of the polypeptide (for example, polylysine), or d) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of the invention.

The tertiary structure of proteins refers to the overall 3-dimensional architecture of a polypeptide chain. The complexity of 3-dimensional structure arises from the intrinsic ability of single covalent bonds to be rotated. Rotation about several such bonds in a linear molecule will produce different nonsuperimpossable 3-dimensional arrangements of the atoms that are generally described as conformations.

Protein conformation is an essential component of protein-protein, protein-substrate, protein-agonist, protein-antagonist interactions. Changes in the component amino acids of protein sequences can result in changes that have little or no effect on the resultant protein conformation. Conversely, changes in the peptide sequences can have effects on the protein conformation resulting in reduced or increased protein-protein, etc. interactions. Such changes and their effects are generally disclosed in Proteins:

Structures and Molecular Properties by Thomas Creightonm W.H. Freeman and Company, New York, 1984 which is hereby incorporated by reference.

"Conformation" and "conformation similarity" when used in this specification and claims refers to a polypeptide's ability (or any other organic or inorganic molecule) to assume a given shape, through folding and the like, so that the shape, or conformation, of the molecule becomes an essential part of it's functionality, sometimes to the exclusion of its chemical makeup. It is generally known that in biological processes two conformational similar molecules may be interchangeable in the process, even the chemically different. "Conformational similarity" refers to the latter interchangeability or substitutability. For example, P400 and P400-derived protein fragments are among the subjects of the invention because they have been shown to bind the Aß protein and render it inactive in fibril formation; it is contemplated that other molecules that are conformationally similar to P400, or any claimed P400 fragment or polypeptide, may be substituted in the claimed method to similarly render the Aß inactive in fibrillogenesis and other amyloid processes. In general it is contemplated that levels of conformational similarity at or above 70% are sufficient to assume homologous functionality in the claimed processes, though reduced levels of conformational similarity may be made to serve as well. Conformational similar levels at or above 90% should provide some level of additional homologue functionality.

Thus, one skilled in the art would envisage that changes can be made to the P400 sequence, or fragments or polypeptides thereof, that would increase, decrease or have no effect on the binding of P400 or fragments thereof, to Aß amyloid. In addition, one skilled in the art would envisage various post-translational modifications such as phosphorylation, glycosylation and the like would alter the binding of P400, P400 fragments or P400 polypeptides to Aß amyloid.

The polypeptides of the present invention include the polypeptides or fragments of P400 and fragments thereof, as well as polypeptides which have at least 70% similarity (preferably 70 % identity) and more preferably a 90% similarity (more preferably a 90% identity) to the polypeptides of P400.

Fragments or portions of the polypeptides or fragments of P400 of the present invention may be employed for producing the corresponding full-length polypeptides by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full length polypeptides.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant procedure, the polypeptides of the present invention may be

glycosylated or may be non glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

Chemical polypeptide synthesis is a rapidly evolving area in the art, and methods of solid phase polypeptide synthesis are well-described in the following references, hereby entirely incorporated by reference (Merrifield, J. Amer. Chem. Soc. 85:2149-2154, 1963; Merrifield, Science 232:341-347, 1986; Fields, Int. J. Polypeptide Prot. Res. 35, 161, 1990).

Recombinant production of P400 polypeptides can be accomplished according to known method steps. Standard reference works seting forth the general principles of recombinant DNA technology include Watson, Molecular Biology of the Gene, Volumes I and II, The Benjamin/Cummings Publishing Company Inc., publisher, Menlo Park, Calif. 1987; Ausubel et al, eds., Current Protocols in Molecular Biology, Wiley Interscience, publisher, New York, N.Y. 1987; 1992; and Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, N.Y. 1989, the entire contents of which references are herein incorporated by reference.

The polypeptides of the present invention may also be utilized as research reagents and materials for discovery of treatments and diagnostics for human diseases.

Antibodies

Antibodies generated against the polypeptides corresponding to specific sequences recognizing the P400 fragments of the present invention which bind Aß or other amyloid proteins can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptides from tissue expressing that polypeptide.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, anti-idiotypic antibodies to antibodies specific for P400 derived protein fragments or polypeptides of the present invention.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen.

A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, Nature 256:495-497,

1975), the trioma technique, the human B-cell hybridoma technique (Kozbor et al, Immunology Today 4:72, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp.77-96, 1985). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in production. Chimeric antibodies and methods for their production are known in the art (ex. Cabilly et al, Proc. Natl. Acad. Sci. U.S.A 81:3273-3277, 1984; Harlow and Lane: Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory 1988).

An anti-idiotypic antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An anti-iodiotypic antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the monoclonal antibody with the monoclonal antibody to which an anti-iodiotypic antibody is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-idiotypic antibody). See, for example, U.S. Patent No. 4,699,880, which is herein incorporated by reference.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')2, which are capable of binding antigen. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al, J. Nucl. Med. 24:316-325, 1983).

The antibodies or fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect P400 or P400-derived fragments in a sample or to detect presence of cells which express a P400 polypeptide of the present invention.This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric or fluorometric detection.

One of the ways in which a P400 antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric, or by visual means. Enzymes which can be used detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase,

glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colometric methods which employ a chromogenic substrate for the enzyme. Detection can be accomplished by colometric methods which employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate with similarly prepared standards (see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory 1988; Ausubel et al, eds., Current Protocols in Molecular Biology, Wiley Interscience, N.Y. 1987, 1992).

Detection may be accomplished using any of a variety of other immunoassays. For example, by radiolabeling of the antibodies or antibody fragments, it is possible to detect R PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work et al, North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, incorporated entirely by reference herein. The radioactive isotope can be detected by such means as the use of a gamma-counter, a scintillation counter or by autoradiography.

It is also possible to label a P400 polypeptide antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, commercially available, e.g., from Molecular Probes, Inc. (Eugene, Oregon, U.S.A.).

The antibody can also be detectably labeled using fluorescence emitting metals such as 152EU, or other of the lanthanide series. These metals can be attached to the antibody using such metal groups as diethylenetriamine pentaacetic acid (EDTA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction, Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt, and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of a P400 fragment of the present invention. In situ detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of a P400 fragment polypeptide but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

In accordance with yet a further aspect of the present invention there are provided antibodies against P400, P400 fragments and/or P400-derived polypeptides which interact with Aß or other amyloid proteins, or derivatives thereof. These antibodies can be used for a number of important diagnostic and/or therapeutic applications as described herein. In one aspect of the invention, polyclonal and/or monoclonal antibodies made against P400, P400 fragments and/or P400-derived polypeptides which bind Aß or other amyloid proteins, may be utilized for Western blot analysis (using standard Western blotting techniques knowledgeable to those skilled in the art) to detect the presence of amyloid protein-binding P400 fragments or amyloid protein-binding P400 polypeptides in human tissues and in tissues of other species. Western blot analysis can also be used to determine the apparent size of each amyloid protein-binding P400 fragment. In addition, Western blotting following by scanning densitometry (known to those skilled in the art) can be used to quantitate and compare levels of each of the P400 fragments in tissue samples. biological fluids or biopsies obtained from individuals with specific diseases (such as the amyloid diseases) in comparison to tissue samples, biological fluids or biopsies obtained from normal individuals or controls. Biological fluids, include, but are not limited to, blood, plasma, serum, cerebrospinal fluid. sputum, saliva, urine and stool.

In yet another aspect of the invention, polyclonal and/or monoclonal antibodies made against P400, P400 fragments and/or P400-derived peptides which bind Aß or other amyloid proteins, can be utilized for immunoprecipitation studies (using standard immunoprecipitation techniques known to one skilled in the art) to detect P400, P400 fragments and/or P400-derived peptides which bind Aß or other amyloid proteins, in tissues, cells and/or biological fluids. Use of the P400, P400 fragments and/or P400-derived peptide antibodies for immunoprecipitation studies can also be quantitated to determine relative levels of P400, P400 fragments and/or P400-derived peptides which interact with Aß or other amyloid proteins, in tissues, cells and/or biological fluids. Quantitative immunoprecipitation can be used to compare levels of P400, P400 fragments and/or P400 amyloid protein-binding peptides in tissue samples,

biological fluids or biopsies obtained from individuals with specific diseases (such as the amyloid diseases) in comparison to tissue samples, biological fluids or biopsies obtained from normal individuals or controls.

Therapeutic Applications

Yet another aspect of the present invention is to make use of P400, P400 fragments and/or P400-derived polypeptides as amyloid inhibitory therapeutic agents. The P400-derived peptide sequences or fragments can be synthesized utilizing standard techniques (ie. using an automated synthesizer). P400, P400 fragments and/or P400-derived peptides which bind Aß or other amyloid proteins, can be used as potential blocking therapeutics for the interaction of P400 in a number of biological processes and diseases (such as in the amyloid diseases described above). In a preferred embodiment, specific peptides made against P400 or fragments thereof may be used to aid in the inhibition of amyloid formation, deposition, accumulation, and /or persistence in a given patient. Likewise, in another preferred embodiment anti-idiotypic antibodies made against P400, P400 fragments and/or P400-derived peptides (as described above) may be given to a human patient as potential blocking antibodies to disrupt continued amyloid formation, deposition, accumulation and/or persistence in the given patient.

Preparations of P400-derived polypeptides for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain axillary agents or excipients which are known in the art. Pharmaceutical compositions such as tablets, pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, can be prepared according to routine methods and are known in the art.

In yet another aspect of the invention, P400, P400 fragments and/or P400-derived peptides may be used as an effective therapy to block amyloid formation, deposition, accumulation and/or persistence as observed in the amyloid diseases. For example, the invention includes a pharmaceutical composition for use in the treatment of amyloidoses comprising a pharmaceutically effective amount of a P400, P400 fragments and/or P400-derived peptide anti-idiotypic antibody and a pharmaceutically acceptable carrier. The compositions may contain P400, P400 fragments and/or P400-derived peptide anti-idiotypic antibody, either unmodified, conjugated to a potentially therapeutic compound, conjugated to a second protein or protein portion or in a recombinant form (ie. chimeric or bispecific P400, P400 fragment and/or P400 polypeptide antibody). The compositions may additionally include other antibodies or conjugates. The antibody compositions of the invention can be administered using conventional modes of

administration including, but not limited to, topical, intravenous, intra-arterial, intraperitoneal, oral, intralymphatic, intramuscular or intralumbar. Intravenous administration is preferred. The compositions of the invention can be a variety of dosage forms, with the preferred form depending upon the mode of administration and the therapeutic application. Optimal dosage and modes of administration for an individual patient can readily be determined by conventional protocols.

P400, P400 fragments and/or P400-derived peptides, or antibodies of the present invention may be administered by any means that achieve their intended purpose, for example, to treat P400 involved pathologies, such as Alzheimer's disease and other amyloid diseases, or other related pathologies, using a P400-derived polypeptide described herein, in the form of a pharmaceutical composition.

For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

A preferred mode of using a P400-derived polypeptide, or antibody pharmaceutical composition of the present invention is by oral administration or intravenous application.

A typical regimen for preventing, suppressing or treating P400-involved pathologies, such as Alzheimer's disease amyloidosis, comprises administration of an effective amount of P400-derived polypeptides, administered over a period of one or several days, up to and including between one week and about 24 months.

It is understood that the dosage of the P400-derived polypeptides of the present invention administered in vivo or in vitro will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation.

The total dose required for each treatment may be administered by multiple doses or in a single dose. A P400-derived polypeptide may be administered alone or in conjunction with other therapeutics directed to P400-involved pathologies, such as Alzheimer's disease or amyloid diseases, as described herein.

Effective amounts of a P400-derived polypeptide or composition, which may also include a P400-fragment derived antibody, are about 0.01μg to about 100mg/kg body weight, and preferably from about 10 μg to about 50 mg/kg body weight, such as 0.05, 0.07, 0.09, 0.1, 0.5, 0.7, 0.9., 1, 2, 5, 10, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mg/kg.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain axillary agents or excipients which are known in the art. Pharmaceutical compositions comprising at least one P400 derived polypeptide, such as 1-10 or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 P400-derived polypeptides, of the present invention may include all compositions wherein the P400- derived polypeptide is contained in an amount effective to achieve its intended purpose. In addition to at least one P400-derived polypeptide, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or axillaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

Pharmaceutical compositions comprising at least one P400-derived polypeptide or antibody may also include suitable solutions for administration intravenously, subcutaneously, dermally, orally, mucosally, rectally or may by injection or orally, and contain from about 0.01 to 99 percent, preferably about 20 to 75 percent of active component (i.e. polypeptide or antibody) together with the excipient. Pharmaceutical compositions for oral administration include pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, and syrups.

The P400, P400 fragments and/or P400-derived peptides for Alzheimer's disease and other central nervous system amyloidoses may be optimized to cross the blood-brain barrier. Methods of introductions include but are not limited to systemic administration, parenteral administration i.e., via an intraperitoneal, intravenous, perioral, subcutaneous, intramuscular, intraarterial, intradermal, intramuscular, intranasal, epidural and oral routes. In a preferred embodiment, P400, P400 fragments and/or P400-derived peptides may be directly administered to the cerebrospinal fluid by intraventricular injection. In a specific embodiment, it may be desirable to administer P400, P400 fragments and/or P400-derived peptides locally to the area or tissue in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by infusion using a cannulae with osmotic pump, by means of a catheter, by means of a suppository, or by means of an implant.

In yet another embodiment P400, P400 fragments and/or P400-derived peptides may be delivered in a controlled release system, such as an osmotic pump. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, ie. the brain, thus requiring only a fraction of the systemic dose.

In yet another aspect of the present invention, peptidomimetic compounds modelled from P400, P400 fragments and/or P400-derived peptides identified as binding Aß or other amyloid proteins, may serve as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in

Alzheimer's disease and other amyloidoses. Peptidomimetic modelling is implemented by standard procedures known to those skilled in the art.

In yet another aspect of the present invention, compounds that mimic the 3 dimensional Aß binding site on P400 using computer modelling, may serve as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses. Design and production of such compounds using computer modeling technologies is implemented by standard procedures known to those skilled in the art.

Recombinant DNA technology, including human gene therapy, has direct applicability to the P400 and their fragments, of this invention. One skilled in the art can take the peptide sequences and create corresponding nucleotide sequences that code for the corresponding peptide sequences. These sequences can be cloned into vectors such as retroviral vectors, and the like. These vectors can, in turn, be transfected into human cells such as hepatocytes or fibroblasts, and the like. Such transfected cells can be introduced into humans to treat amyloid diseases. Alternatively, the genes can be introduced into the patients directly. The basic techniques of recombinant DNA technology are known to those of ordinary skill in the art and are disclosed in Recombinant DNA Second Edition, Watson, et al., W.H. Freeman and Company, New York, 1992, which is hereby incorporated by reference.

Diagnostic Applications

Another aspect of the invention is to provide polyclonal and/or monoclonal antibodies against P400, P400 fragments and/or P400-derived peptides which bind Aß or other amyloid proteins, which would be utilized to specifically detect P400. P400 fragments and/or P400-derived peptides in human tissues and/or biological fluids. In one preferred embodiment, polyclonal or monoclonal antibodies made against a peptide portion or fragment of P400, can be used to detect and quantify P400, P400 fragments and/or P400-derived peptides in human tissues and/or biological fluids. Polyclonal and/or monoclonal peptide antibodies can also be utilized to specifically detect P400 fragments and/or P400-derived polypeptides in human tissues and/or biological fluids. In a preferred embodiment, a polyclonal or monoclonal antibody made specifically against a peptide portion of P400 which binds Aß (as described herein), can be used to detect and quantify this P400 fragment in human tissues and/or biological fluids. In another preferred embodiment, a polyclonal or monoclonal antibody made specifically against a peptide portion or fragment of P400 which is present in human biological fluids and binds Aß (as described herein), can be used to detect and quantify this P400 fragment in human tissues and/or biological fluids.

For detection of P400 fragments and/or P400-derived polypeptides described above in human

tissues, cells, and/or in cell culture, the polyclonal and/or monoclonal antibodies can be utilized using standard immunohistochemical and immunocytochemical techniques, known to one skilled in the art.

For detection and quantitation of P400, P400 fragments and/or P400-derived peptides in biological fluids, including cerebrospinal fluid, blood, plasma, serum, urine, sputum, and/or stool, various types of ELISA assays can be utilized, known to one skilled in the art. An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier, and a quantity of detectable labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid phase antibody, antigen, and labeled antibody.

In a preferred embodiment, a "sandwich" type of ELISA can be used. Using this preferred method a pilot study is first implemented to determine the quantity of binding of each P400-fragment monoclonal antibody to microtiter wells. Once this is determined, aliquots (usually in 40 µl of TBS; pH 7.4) of the specific P400-fragment antibody are allowed to bind overnight to microtiter wells (Maxisorb C plate from Nunc) at 4oC. A series of blank wells not containing any P400-fragment specific monoclonal antibody are also utilized as controls. The next day, non-bound monoclonal antibody is shaken off the microtiter wells. All of the microtiter wells (including the blank wells) are then blocked by incubating for 2 hours with 300 µl of Tris-buffered saline containing 0.05% Tween-20 (TTBS) plus 2% bovine serum albumin, followed by 5 rinses with TTBS. 200 µl of cerebrospinal fluid, blood, plasma, serum, urine, sputum, and/or stool and/or any other type of biological sample is then diluted (to be determined empirically) in TTBS containing 2% bovine serum albumin and placed in wells (in triplicate) containing bound P400 fragment antibody (or blank) and incubated for 2 hours at room temperature. The wells are then washed 5 times with TTBS. A second biotinylated-monoclonal antibody against the same P400-derived fragment (but which is against a different epitope) is then added to each well (usually in 40 µl of TBS; pH 7.4) and allowed to bind for 2 hours at room temperature to any P400-fragment captured by the first antibody. Following incubation, the wells are washed 5 times with TTBS. Bound materials are then detected by incubating with 100 µl of peroxidase-avidin complex (1:250 dilution in TTBS with 0.1% BSA) for 1 hour on a rotary shaker. After 5 washes with TTBS, a substrate solution (100 µl, OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO, USA) is added and allowed to develop significant color (usually 8-10 minutes). The reaction is stopped with 50 µl of 4N sulfuric acid and read on a standard spectrophotometer at 490 nm. This ELISA can be utilized to determine differences in specific P400 fragments (and/or Aß-binding P400 fragments) in biological fluids which can serve as a diagnostic marker to follow the progression on a live patient during the progression of disease (i.e. monitoring of amyloid disease as an

example). In addition, quantitative changes in P400 fragments can also serve as a prognostic indicator monitoring how a live patient will respond to treatment which targets a given amyloid disease such as Alzheimer's disease. Such assays can be provided in a kit form.

A competition assay may also be employed wherein antibodies specific to P400, P400 fragments and/or P400-derived peptides are attached to a solid support and labeled P400, P400 fragments and/or P400-derived peptides and a sample derived from a host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to the quantity of P400, P400 fragments and/or P400-derived peptides in the sample. This standard technique is known to one skilled in the art.

Another object of the present invention is to use P400, P400 fragments and/or P400-derived peptides, in conjunction with P400, P400 fragment and/or P400 derived peptide antibodies, in an ELISA assay to detect potential P400, P400 fragment and/or P400-derived peptide autoantibodies in human biological fluids. Such a diagnostic assay may be produced in a kit form. In a preferred embodiment, peptides containing the sequences of P400, P400 fragments and/or P400-derived peptides, as well as polypeptides which have at least 70% similarity (preferably 70 % identity) and more preferably a 90% similarity (more preferably a 90% identity) to the polypeptides described above, will be used to initially bind to microtiter wells in an ELISA plate. A pilot study is first implemented to determine the quantity of binding of each P400 fragment polypeptide to microtiter wells. Once this is determined, aliquots (usually 1-2μg in 40 μl of TBS; pH 7.4) of specific P400 fragment polypeptides (as described herein) are allowed to bind overnight to microtiter wells (Maxisorb C plate from Nunc) at 4°C. All the microtiter wells (including blank wells without the P400 fragment polypeptides) are blocked by incubating for 2 hours with 300 μl of Tris-buffered saline (pH 7.4) with 0.05% Tween-20 (TTBS), containing 2% albumin. This is followed by 5 rinses with TTBS. The patients' biological fluids (i.e., cerebrospinal fluid, blood, plasma, serum, sputum, urine, and/or stool) are then utilized and 200 μl are diluted (to be determined empirically) with TTBS containing 2% bovine serum albumin, and placed in microtiter wells (in triplicate) containing a specific P400 fragment polypeptide or blank wells (which do not contain peptide), and are incubated at 1.5 hours at room temperature. Any autoantibodies present in the biological fluids against the P400 fragment will bind to the substrate bound P400 fragment polypeptide (or fragments thereof). The wells are then rinsed by washing 5 times with TTBS. 100 μl of biotinylated polyclonal goat anti-human IgGs (Sigma Chemical company, St. Louis, MO, USA), diluted 1:500 in TTBS with 0.1% bovine serum albumin, is then aliquoted into each well. Bound materials are detected by incubating with 100 μl of peroxidase-avidin complex (1:250 dilution in TTBS with 0.1% bovine serum albumin) for 1 hour on a rotary shaker. Following 5 washes with TTBS, substrate solution (100 μl, OPD Sigma Fast from Sigma

Chemical Company, St. Louis, MO, USA) is added and allowed to develop significant color (usually 8-10 minutes). The reaction is stopped with 50 µl of 4N sulfuric acid added to each well and read on a standard spectrophotometer at 490 nm. This assay system can be utilized to not only detect the presence of autoantibodies against P400 fragments in biological fluids, but also to monitor the progression of disease by following elevation or diminution of P400 fragment autoantibody levels. It is believed that patients demonstrating excessive P400 fragment formation, deposition, accumulation and/or persistence as may be observed in the amyloid diseases will also carry autoantibodies against the P400 fragments in their biological fluids. Various ELISA assay systems, knowledgeable to those skilled in the art, can be used to accurately monitor the degree of P400 fragments in biological fluids as a potential diagnostic indicator and prognostic marker for patients during the progression of disease (i.e. monitoring of an amyloid disease for example). Such assays can be provided in a kit form. In addition, quantitative changes in P400 fragment autoantibody levels can also serve as a prognostic indicator monitoring how a live patient will respond to treatment which targets a given amyloid disease.

Other diagnostic methods utilizing the invention include diagnostic assays for measuring altered levels of P400, P400 fragments and/or P400-derived peptides in various tissues compared to normal control tissue samples. Assays used to detect levels of P400, P400 fragments and/or P400-derived peptides in a sample derived from a host are well-known to those skilled in the art and included radioimmunoassays, competitive-binding assays, Western blot analysis and preferably ELISA assays (as described above).

Yet another aspect of the present invention is to use the antibodies recognizing P400, P400 fragments and/or P400-derived peptides for labellings, for example, with a radionucleotide, for radioimaging or radioguided surgery, for in vivo diagnosis, and/or for in vitro diagnosis. In one preferred embodiment, radiolabelled peptides or antibodies made (by one skilled in the art) against P400, P400 fragments and/or P400-derived peptides may be used as minimally invasive techniques to locate P400, P400 fragments and/or P400-derived peptides, and concurrent amyloid deposits in a living patient. These same imaging techniques could then be used at regular intervals (i.e. every 6 months) to monitor the progression of the amyloid disease by following the specific levels of P400, P400 fragments and/or P400-derived peptides.

Yet another aspect of the present invention is to provide a method which can evaluate a compound's ability to alter (diminish or eliminate) the affinity of a given amyloid protein (as described herein) or amyloid precursor protein, to P400, P400 fragments and/or P400-derived peptides. By providing a method of identifying compounds which affect the binding of amyloid proteins, or amyloid precursor proteins to such P400 derived fragments, the present invention is also useful in identifying

compounds which can prevent or impair such binding interaction. Thus, compounds can be identified which specifically affect an event linked with the amyloid formation, amyloid deposition, and/or amyloid persistence condition associated with Alzheimer's disease and other amyloid diseases as described herein.

According to one aspect of the invention, to identify for compounds which allow the interaction of amyloid proteins or precursor proteins to P400-derived fragments or P400 polypeptides, either amyloid or P400 fragments are immobilized, and the other of the two is maintained as a free entity. The free entity is contacted with the immobilized entity in the presence of a test compound for a period of time sufficient to allow binding of the free entity to the immobilized entity, after which the unbound free entity is removed. Using antibodies which recognize the free entity, or other means to detect the presence of bound components, the amount of free entity bound to immobilized entity can be measured. By performing this assay in the presence of a series of known concentrations of test compound and, as a control, the complete absence of test compound, the effectiveness of the test compound to allow binding of free entity to immobilized entity can be determined and a quantitative determination of the effect of the test compound on the affinity of free entity to immobilized entity can be made. By comparing the binding affinity of the amyloid-P400 fragment complex in the presence of a test compound to the binding affinity of the amyloid-P400 fragment complex in the absence of a test compound, the ability of the test compound to modulate the binding can be determined.

In the case in which the amyloid is immobilized, it is contacted with free P400 derived fragments or polypeptides, in the presence of a series of concentrations of test compound. As a control, immobilized amyloid is contacted with free P400-derived polypeptides, or fragments thereof in the absence of the test compound. Using a series of concentrations of P400-derived polypeptides, the dissociation constant (K_d) or other indicators of binding affinity of amyloid-P400 fragment binding can be determined. In the assay, after the P400-derived polypeptides or fragments thereof is placed in contact with the immobilized amyloid for a sufficient time to allow binding, the unbound P400 polypeptides are removed. Subsequently, the level of P400 fragment-amyloid binding can be observed. One method uses P400-derived fragment antibodies, as described in the invention, to detect the amount of specific P400 fragments bound to the amyloid or the amount of free P400 fragments remaining in solution. This information is used to determine first qualitatively whether or not the test compound can allow continued binding between P400-derived fragments and amyloid. Secondly, the data collected from assays performed using a series of test compounds at various concentrations, can be used to measure quantitatively the binding affinity of the P400 fragment-amyloid complex and thereby determine the effect of the test compound on the affinity between P400 fragments and amyloid. Using this information, compounds can be identified which do not modulate the binding of specific P400 fragments to amyloid and thereby allow the P400 fragments to

reduce the amyloid formation, deposition, accumulation and/or persistence, and the subsequent development and persistence of amyloidosis.

Therefore a kit for practicing a method for identifying compounds useful which do not alter P400, P400 fragments and/or P400-derived peptides to an immobilized amyloid protein, said kit comprising a) a first container having amyloid protein immobilized upon the inner surface, b) a second container which contains P400, P400 fragments and/or P400-derived peptides dissolved in solution, c) a third container which contains antibodies specific for said P400, P400 fragments and/or P400-derived peptides, said antibodies dissolved in solution, and d) a fourth container which contains labeled antibodies specific for P400, P400 fragments and/or P400-derived peptides, said antibodies dissolved in solution.

CLAIMS

We claim:

1. A pharmacological agent for binding to a beta-amyloid protein present in a biological fluid, wherein the pharmacological agent comprises a therapeutically effective amount of a component of the biological fluid.

2. The pharmacological agent of claim 1 comprising a protein of molecular weight of approximately 350 to 450 kilodaltons (P400).

3. The pharmacological agent of claim 1 comprising P400, P400-derived protein fragments, or P400-derived polypeptides.

4. The pharmacological agent of claim 1 wherein the biological fluid is selected from a group of biological fluids consisting of blood, plasma, serum, cerebrospinal fluid, sputum, saliva, urine and stool.

5. The pharmacological agent of claim 1 wherein the biological fluid is of human origin.

6. A method of detection and/or quantification of P400, P400-derived protein fragments or P400-derived polypeptides, in biological fluids wherein the method comprises the step of ligand blotting using biotinylated-beta-amyloid protein.

7. The method of claim 6 whereby the method of detection and/or quantification further comprises the following steps: a) use of SDS-PAGE to separate proteins present in biological fluids, b) transferring of said proteins to membranes used for ligand blotting, c) probing of transferred proteins using a beta-amyloid protein that has been biotinylated, d) detection of P400, P400-derived protein fragments or P400-derived polypeptides, using such ligand blotting methods, and e) quantification of levels of P400, P400-derived protein fragments or P400-derived polypeptides, in a sample using scanning densitometry or other detection methods.

8. The method of claim 6 further comprising the use of antibodies generated against P400, P400-derived protein fragments or P400-derived polypeptides.

9. The method of claim 8 further comprising ELISA based use of the antibodies for the detection of P400, P400-derived protein fragments or P400-derived polypeptides, in biological samples.

10. The ELISA method of claim 9 further comprising of the following steps: a) allowing a P400, P400-derived protein fragment or P400-derived polypeptide monoclonal antibody to bind to microtiter wells for sufficient time to allow said binding, b) adding a quantity of biological fluid , c) adding to each well a second labeled monoclonal antibody against the same P400, P400-derived protein fragments or P400-derived polypeptides, but which is against a different epitope, to bind to any P400, P400-derived protein fragments or P400-derived polypeptides captured by the first antibody, d) detection of bound materials by incubating with a substrate until a significant color reaction is allowed to develop.

11. The method of claim 6 as applied to diagnose a disease or a susceptibility to a disease related to the levels of P400, P400-derived protein fragments or P400-derived polypeptides. wherein elevated or diminished levels of P400, P400-derived protein fragments or P400-derived polypeptides will be indicative of the presence of a disease, susceptibility to a disease, and progression of said disease.

12. The method of claim 11 wherein said disease is selected from the group of disease consisting of Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis of the Dutch type, and another disease that involves the deposition of a beta-amyloid protein.

13. A method for the treatment of a patient having an identified clinical need to interfere with the pathological effects of amyloid, the method comprising administering to a patient a therapeutically effective amount of P400, P400-derived protein fragments, or P400-derived polypeptides.

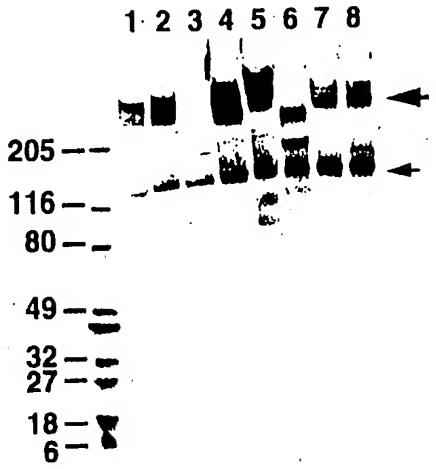

FIG. 1

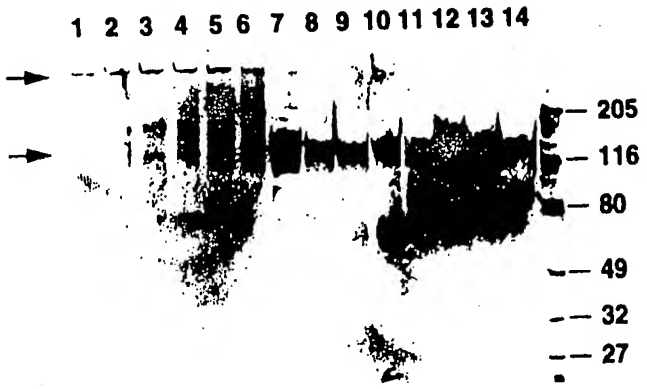

FIG. 2

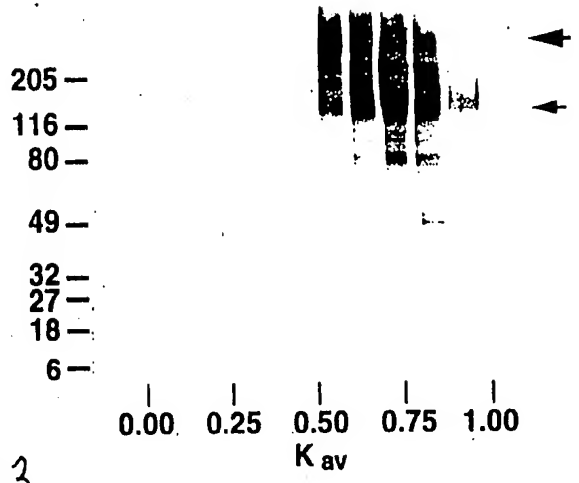

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06878

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C07K 5/00, 16/0; A61K 38/00
US CL :514/2; 530/300, 395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/300, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Author search

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P — Y,P	US 5,958,883 A (SNOW) 28 September 1999 (28.09.99), see entire document.	1-3 --- 4-13
X --- Y	US 5,164,295 A (KISILEVSKY et al.) 17 November 1992 (17.11.92), see entire document.	1-3 --- 4-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 JULY 2000	Date of mailing of the international search report 15 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX SHARON L. TURNER Telephone No. (703) 308-0196

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